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## The catabolism of L-tyrosine by an *Arthrobacter* sp.<sup>1</sup>

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An *Arthrobacter* sp. metabolizes L-tyrosine by a pathway involving 3,4-dihydroxyphenylacetate as a key intermediate. *p*-Hydroxyphenylpyruvate is formed from tyrosine by an aminotransferase specifically requiring  $\alpha$ -ketoglutarate for activity, and is then converted to *p*-hydroxyphenylacetate by an oxidative decarboxylation. *p*-Hydroxyphenylacetaldehyde is not an intermediate in the formation of *p*-hydroxyphenylacetate. Extracts of the bacterium oxidize 3,4-dihydroxyphenylacetate to  $\delta$ -carboxymethyl- $\alpha$ -hydroxymuconic acid which, when supplemented with 2 mol of diphosphopyridine dinucleotide, results in the production of stoichiometric amounts of succinate and pyruvate.

BLAKLEY E. R. 1977. The catabolism of L-tyrosine by an *Arthrobacter* sp. Can. J. Microbiol. 23: 1128-1139.

Un *Arthrobacter* sp. métabolise la L-tyrosine par une voie qui implique le 3,4-dihydroxyphénylacétate comme un intermédiaire majeur. Le *p*-hydroxyphénylpyruvate est formé à partir de la tyrosine par une aminotransférase dont l'activité nécessite spécifiquement la présence de  $\alpha$ -cétoglutarate; par la suite le *p*-hydroxyphénylpyruvate est transformé en *p*-hydroxyphénylacétate par une décarboxylation oxydative. Le *p*-hydroxyphénylacétaldéhyde n'est pas un intermédiaire dans la formation du *p*-hydroxyphénylacétate. Des extraits cellulaires oxydent le 3,4-dihydroxyphénylacétate en acide  $\delta$ -carboxyméthyl- $\alpha$ -hydroxymuconique, lequel, en présence de 2 mol de diphosphopyridine dinucléotide forme des quantités stœchiométriques de succinate et de pyruvate.

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### Introduction

The major pathway for the catabolism of L-tyrosine in mammals, and probably in microorganisms, involves homogentisate as a key intermediate (13, 22). Evidence in support of the homogentisate pathway in microorganisms comes from the identification of homogentisate in the culture medium after growth on tyrosine (9, 16), the ability of tyrosine-grown cells to metabolize homogentisate and *p*-hydroxyphenylpyruvate (9, 18), and the presence of high levels of homogentisate-1,2-dioxygenase (EC 1.13.11.5) in extracts of cells grown on tyrosine (18, 27). The enzymatic conversion of tyrosine to homogentisate has been described for mammalian systems but not for microbial systems.

Alternate pathways for the degradation of tyrosine have been summarized by Towers and Subba Rao (29).

During a survey of microorganisms which could metabolize tyrosine, an *Arthrobacter* sp. was found, which degraded tyrosine by a pathway involving 3,4-dihydroxyphenylacetate (homopro-

tocatechuate) as an intermediate. This paper describes the experiments designed to elucidate the pathway for the degradation of L-tyrosine by the *Arthrobacter*.<sup>2</sup>

### Materials and Methods

#### Growth of Organism

The organism (designated PRL W15) was isolated from soil and identified as a strain of *Arthrobacter globiformis* (5). Small amounts of cells were produced by growing the organism at 30°C in 100 ml of salts medium (5) supplemented with 0.1% L-tyrosine in a 500-ml Erlenmeyer flask on a rotary shaker (250 rpm, eccentricity 1 in.). The cells were collected and washed with water by centrifugation. Larger amounts of cells were obtained by growing the cells in 20 l of salts medium containing 0.1% L-tyrosine and 0.1% gluconate contained in a Microferm Laboratory Fermentor (New Brunswick Scientific Co.). The medium was inoculated with 500 ml of cells grown overnight on 0.2% gluconate, and grown for 18 h at 30°C with stirring at 200 rpm with an airflow of 4 l per minute. The cells were collected and washed with water by centrifugation then stored at -30°C.

#### Preparation and Treatment of Extracts

Extracts of cells were prepared by treating a suspension of one volume of cells in two volumes of buffer with a

<sup>1</sup>NRCC N. 15969.

<sup>2</sup>Some of the results were presented at the 18th Annual Meeting, Can. Fed. Biol. Soc., Winnipeg, Man. 1975.

Raytheon 10-kcycle sonic oscillator for 10 min at 5°C, followed by centrifugation at  $45\,000 \times g$  for 20 min. Extracts prepared in this manner are referred to as 'crude extracts.' The following buffer systems were used: *buffer A*, 0.05 M tris(hydroxymethyl)aminomethane (Tris) adjusted with HCl to pH 7.5; *buffer B*, 0.05 M Tris-HCl containing  $10^{-4}$  M thiamine pyrophosphate (TPP),  $10^{-4}$  M  $MgCl_2$ ,  $5 \times 10^{-3}$  M mercaptoethanol, pH 7.5; *buffer C*, 0.05 M potassium phosphate,  $10^{-4}$  M TPP,  $10^{-4}$  M  $MgCl_2$ ,  $5 \times 10^{-3}$  M mercaptoethanol, pH 7.5. Most of the experiments involved the use of buffer A, but for studies of *p*-hydroxyphenylpyruvate oxidase, buffers B or C were used.

'Crude extracts' were centrifuged at  $150\,000 \times g$  for 2 h in a Beckman model L preparative ultracentrifuge to remove reduced nicotinic adenine dinucleotide (NADH) oxidase activity. These preparations are termed 'ultracentrifuged extracts.'

The ultracentrifuged extract was treated with 0.1 volume of 2% protamine sulfate and the clear supernatant was fractionated by the addition of saturated ammonium sulfate, followed by solid ammonium sulfate to obtain the desired salt concentration. The protein precipitates were collected by centrifugation and dissolved in an amount of buffer approximately equivalent to  $\frac{1}{2}$  of the original volume.

#### Manometric Studies

The activities of intact cells toward various substrates were determined in the Warburg apparatus by measuring the rates of oxygen consumption at 30°C. Each flask contained 100  $\mu$ mol of phosphate buffer, pH 7.0, 30 mg cells (wet weight), 180  $\mu$ g of chloramphenicol, and 10  $\mu$ mol of substrate in a final volume of 3.0 ml. The center well contained 0.2 ml of 20% KOH to absorb carbon dioxide. For most of the experiments, the substrate was added from the sidearm at zero time, but when the experiments involved the addition of tyrosine, because of its low solubility, the cells were preincubated with chloramphenicol and added from the sidearm, while the substrates were contained in the main compartment of the Warburg flask.

The consumptions of oxygen by cell extracts were determined in the Warburg apparatus at 30°C. Each flask contained 100  $\mu$ mol of Tris buffer pH 7.0, 1.0 ml of extract, substrate, and cofactors as indicated in the discussion of results. The center well contained 0.2 ml of 20% KOH. For some experiments the Tris buffer was replaced with 0.0125 M  $NaHCO_3$ . The production of carbon dioxide during oxygen consumption was determined by the direct method of Warburg (31).

#### Assays for Enzyme Activities

All enzyme assays were carried out at room temperature (about 28°C).

##### (a) L-Tyrosine Aminotransferase (EC 2.6.1.5)

This was determined by the enol-borate method of Lin *et al.* (19) or by the method of Diamondstone (10). Diethyldithiocarbamate had no effect on the reaction and was not included in the reaction mixture.

##### (b) *p*-Hydroxyphenylpyruvate Oxidase

This was assayed by determining the disappearance of *p*-hydroxyphenylpyruvate from a reaction mixture. During the early studies, the concentration of *p*-hydroxyphenylpyruvate was determined by spectrophotometric

measurement of the enol-borate complex (19). A more convenient assay involved the spectrophotometric measurement of *p*-hydroxyphenylpyruvate-2,4-dinitrophenylhydrazone in alkaline solution. The reaction mixture contained 200  $\mu$ mol of phosphate buffer, pH 7.5, 4  $\mu$ mol of TPP, 10  $\mu$ mol of magnesium chloride, 2  $\mu$ mol of reduced glutathione (GSH), 0.01  $\mu$ mol of flavine adenine dinucleotide (FAD), 0.2  $\mu$ mol of *p*-hydroxyphenylpyruvate, enzyme, and water to a final volume of 3.0 ml. The reaction mixture was incubated for 20 min at 28°C and stopped by the addition of 0.2 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. After 30 min at room temperature (28°C) the samples were mixed with 0.2 ml of 10 N NaOH, clarified by centrifugation, and the optical density determined at 445 nm using a Beckman D.U. spectrophotometer. The concentration of substrate at zero time was determined in samples to which the enzyme solution was added after the addition of acidic 2,4-dinitrophenylhydrazine reagent. The values obtained were corrected by blank values obtained from reaction mixtures without substrate. One unit of enzyme catalyzed the disappearance of 1  $\mu$ mol of substrate per minute.

##### (c) *p*-Hydroxyphenylacetate Hydroxylase

This was detected in extracts by measuring the decrease in optical density at 340 nm. The reaction mixture contained 100  $\mu$ mol of Tris buffer, pH 7.5, 0.5  $\mu$ mol of reduced nicotinic adenine dinucleotide phosphate (NADPH), 5  $\mu$ mol of *p*-hydroxyphenylacetate, enzyme, and water to 3.0 ml. Because of interferences due to the presence of other enzyme activities in the enzyme preparations, the assay was suitable only for a qualitative demonstration of *p*-hydroxyphenylacetate hydroxylase activity.

##### (d) 3,4-Dihydroxyphenylacetate 2,3-Dioxygenase (EC 1.13.11.15)

This was determined by measuring the rate of production of  $\delta$ -carboxymethyl- $\alpha$ -hydroxymuconic semialdehyde (CMHS) at 380 nm. The reaction mixture contained 100  $\mu$ mol of Tris buffer, pH 8.5, 0.4  $\mu$ mol of 3,4-dihydroxyphenylacetate dissolved in water, enzyme, and water to 3.0 ml. One unit of enzyme produced 1  $\mu$ mol of CMHS per minute using  $E = 3.8 \times 10^4 M^{-1} cm^{-1}$  as the molar extinction coefficient of CMHS (17).

##### (e) CMHS dehydrogenase

This was determined by measuring the disappearance of CMHS at 380 nm. The reaction mixture contained 100  $\mu$ mol of Tris buffer, pH 8.5, 0.5  $\mu$ mol of CMHS, 2  $\mu$ mol of nicotinic adenine dinucleotide (NAD<sup>+</sup>), 5  $\mu$ mol of  $MgCl_2$ , enzyme, and water to 3.0 ml. One unit of activity catalyzed the oxidation of 1  $\mu$ mol of CMHS per minute. For some experiments the formation of reduced NAD<sup>+</sup> was determined at 340 nm. However, the measurement at 340 nm was not satisfactory for the estimation of enzyme activity because of interference from CMHS.

##### (f) Succinic Semialdehyde Dehydrogenase (EC 1.2.1.16)

This was determined by measuring the rate of reduction of nicotinic adenine dinucleotide phosphate (NADP<sup>+</sup>) at 340 nm. The reaction mixture contained 100  $\mu$ mol of Tris buffer, pH 7.0, 5  $\mu$ mol of succinic semialdehyde, 5  $\mu$ mol of NADP<sup>+</sup>, enzyme, and water to 3.0 ml. One unit of activity catalyzed the production of 1  $\mu$ mol NADPH per minute.

### Chemical Assays

Protein was determined by the method of Lowry *et al.* (21) using serum albumin as a standard. 3,4-Dihydroxyphenylacetic acid was determined by the method of Arnov (2), *p*-hydroxyphenylacetic acid by reaction with diazotized *p*-nitroaniline as described by Bray and Thorpe (7). CHMS was assayed at 380 nm in sodium carbonate solution (6). Tyrosine was determined by the method of Udenfriend and Cooper (30) and *p*-hydroxyphenylpyruvate by the method of Diamondstone (10) or Lin *et al.* (19).

Hydrogen peroxide was detected by adding to a reaction mixture an equal volume of *o*-dianisidine reagent of the following composition: 10 mg *o*-dianisidine hydrochloride, 18.6 mg ethylenediaminetetraacetate (EDTA), 5.0 ml 0.1% peroxidase in 0.05 M sodium acetate, pH 5.0, 1.0 ml 10% Triton X-100, in 50 ml 0.05 M sodium acetate pH 5.0 (32).

### Source of Chemical Compounds

Most of the compounds used in this study were obtained from commercial supply companies and were purified by recrystallization as required. Succinic semialdehyde was prepared by (a modification of) the method of Jacoby (15) or by boiling an aqueous solution of aconic acid (8). In the latter procedure the reaction mixture was sampled during the hydrolysis and assayed for succinic semialdehyde by the procedure of Taberner *et al.* (28). The reaction was complete after 5 h. The aqueous solutions of succinic semialdehyde were stored in small portions at  $-20^{\circ}\text{C}$ . *p*-Hydroxyphenylacetaldehyde was prepared by the rearrangement of synephrine (23), isolated, and stored as the bisulfite addition complex. Aqueous solutions of *p*-hydroxyphenylacetaldehyde were prepared from the bisulfite compound (23) and only freshly prepared solutions were used. CHMS was prepared by the enzymatic oxidation of 3,4-dihydroxyphenylacetate as described below.

### Preparation of $\delta$ -Carboxymethyl- $\alpha$ -Hydroxymuconic Semialdehyde (CHMS)

Dialyzed crude extracts, or extracts heated to  $55^{\circ}\text{C}$  for 5 min, were found satisfactory for the preparation of CHMS. In a typical experimental preparation, 100 ml of extract, pH 7.0 (10 mg protein per millilitre) was added to 200 ml of 0.015 M Tris buffer containing 336 mg 3,4-dihydroxyphenylacetic acid (2.0 mmol), pH 7.0, in a beaker with a bottom surface large enough to provide a liquid layer about 2.0 cm deep. The reaction was stirred with a magnetic stirrer and maintained at pH 7.2 by the addition of 1.0 N NaOH from a microburette. Samples taken periodically during the reaction period were acidified, centrifuged, and assayed for 3,4-dihydroxyphenylacetic acid (2) and CHMS (6). When the disappearance of 3,4-dihydroxyphenylacetate was complete (2–3 h) the mixture was acidified to pH 1 with concentrated HCl, saturated with NaCl, and continuously extracted with ether for 20 h. The ether extract was dried with anhydrous  $\text{MgSO}_4$  and the volume reduced at  $35^{\circ}$  on a rotary evaporator until the yellow product began to separate. The solution was stored at  $0^{\circ}\text{C}$  overnight and the product collected by filtration.

### Isolation and Identification of Products of Reactions with Cell Extracts

The product of the reaction with tyrosine was isolated

from a reaction mixture containing 250  $\mu\text{mol}$  of tyrosine, 12 mmol of phosphate, 2.4  $\mu\text{mol}$  of pyridoxal phosphate (PP), 600  $\mu\text{mol}$  of  $\alpha$ -ketoglutarate, and 6 ml of crude extract (about 50 mg of protein, prepared in buffer A) in a total volume of 60 ml, pH 8.0. The reaction was started by the addition of  $\alpha$ -ketoglutarate after preincubation of the other reaction components by stirring for 20 min at room temperature ( $28^{\circ}\text{C}$ ). After the addition of  $\alpha$ -ketoglutarate, stirring was continued for  $3\frac{1}{2}$  h and appropriate samples were removed and assayed for *p*-hydroxyphenylpyruvate by the method of Diamondstone (10). At completion of the reaction period, 1.5 ml of reaction mixture was treated with 0.5 ml of 20% metaphosphoric acid and the clear supernatant was assayed for *p*-hydroxyphenylpyruvate by the method of Lin *et al.* (19). The remaining reaction mixture was adjusted to pH 2 with HCl and the precipitated protein removed by centrifugation. The clear supernatant was saturated with NaCl and extracted with ether ( $3 \times 100$  ml). After the ether extract was dried with anhydrous  $\text{MgSO}_4$  and evaporated to dryness, a portion of the product was examined by paper chromatography, and the remainder was treated with 2,4-dinitrophenylhydrazine. The hydrazones were extracted into ethyl acetate and partially purified by extraction into 10%  $\text{Na}_2\text{CO}_3$ , acidification, and reextraction into ethyl acetate. The hydrazones of the product and of an authentic sample of *p*-hydroxyphenylpyruvate were compared by paper and thin-layer chromatography.

The product of the reaction with *p*-hydroxyphenylpyruvate was extracted with ether from acidified reaction mixtures following manometric studies carried out in the Warburg apparatus. Each flask contained 200  $\mu\text{mol}$  of phosphate buffer, pH 6.5, 4  $\mu\text{mol}$  of thiamine pyrophosphate, 10  $\mu\text{mol}$  of  $\text{MgCl}_2$ , 0.01  $\mu\text{mol}$  of flavine adenine dinucleotide, 2  $\mu\text{mol}$  of reduced glutathione, 10  $\mu\text{mol}$  of substrate, and 1.0 ml of crude extract (about 10 mg protein, prepared in buffer C) in a final volume of 3.0 ml. When the reactions were completed, as indicated by the cessation of oxygen consumption, the contents of several flasks were combined with HCl and the protein removed by centrifugation. The product was extracted from the supernatant with ether; the ether extract was dried with anhydrous  $\text{MgSO}_4$  and concentrated to a small volume. A portion of the product was examined by thin-layer chromatography, while the remainder was treated with *N,O*-bis(trimethylsilyl)acetamide (BSA) and examined by gas-liquid chromatography. Reaction mixtures containing no substrate were also treated in the same manner.

The reactions of 3,4-dihydroxyphenylacetate with cell extracts in the presence of  $\text{NAD}^+$ , carried out in the Warburg apparatus, were examined for reaction products. The reaction mixtures contained 10  $\mu\text{mol}$  of 3,4-dihydroxyphenylacetate, 15  $\mu\text{mol}$  of  $\text{NAD}^+$ , 100  $\mu\text{mol}$  of phosphate buffer, pH 7.0, and 1 ml of crude extract in a total volume of 3.0 ml. After the consumption of oxygen ceased, the contents of four flasks were combined, acidified to pH 1 with 6 N HCl, and centrifuged. The supernatants were (A) treated with about 3 ml of 2,4-dinitrophenylhydrazine per ml of 3,4-dihydroxyphenylacetate, (B) saturated with NaCl and extracted continuously with ether for 20 h, or (C) treated with 10 mg of hydroxylamine hydrochloride to react with pyruvate (4), saturated with NaCl, and extracted continuously

with ether for 20 h. The 2,4-dinitrophenylhydrazones formed in A were extracted into ethylacetate, and purified by extraction into 10% aqueous sodium carbonate, acidification, and reextraction into ethyl acetate. The product was chromatographed on paper and on thin-layer silica gel and compared with hydrazones prepared from pyruvate,  $\alpha$ -ketoglutarate (KG), and succinic semialdehyde.

The ether extracts from B and C were dried with anhydrous  $\text{MgSO}_4$  and evaporated to near dryness. The sample from B was examined by paper and thin-layer chromatography while the sample from C was converted to silyl derivatives with 0.5 ml  $N,O$ -bis(trimethylsilyl)-acetamide (BSA). A measured portion of the reaction mixture was examined by gas-liquid chromatography, and the results compared with controls containing succinate and pyruvate in amounts equimolar to 3,4-dihydroxyphenylacetate.

#### Chromatography

Reaction products were examined at room temperature by ascending chromatography on No. 54 Whatman filter paper (W) or by thin layer (TL) on silica-coated plastic sheets (Eastman Kodak 6060). Several solvent systems were used during the study. Some of the more successful systems (v/v) were as follows: for the 2,4-dinitrophenylhydrazones:

- (A) chloroform : methanol : water : formic acid (100:10:9.6:0.4);
- (B) ethylacetate (saturated with 0.1 N  $\text{NaHCO}_3$ ) : methanol (5:1);
- (C) petroleum ether (bp 35–60°) : ether : formic acid (100:100:6);  
for the aliphatic acids:
- (D) ethanol : ammonia : water (80:5:15);
- (E) ether : formic acid : water (5:2:1);  
and for aromatic acids:
- (F) *n*-butanol : acetic acid : water (4:1:1);
- (G) benzene : hexane : acetic acid (25:75:5);
- (H) petroleum ether (bp 35–60°) : ether : formic acid (125:75:6);
- (I) toluene : ethyl formate : formic acid (50:25:5);
- (J) isopropanol : ammonia : water (8:1:1).

Phenolic compounds were detected by spraying with diazotized *p*-nitroaniline (7) or Folin-Ciocalteu reagent (Fisher Scientific Co.). 2,4-Dinitrophenylhydrazones were detected by spraying with 5% sodium carbonate. Acids were detected by spraying with an aqueous solution of 2,6-dichlorophenolindophenol (1%). Gas-liquid chromatography was carried out using 6 ft  $\times$   $\frac{1}{8}$  in. stainless steel columns containing 10% SE30 on chromosorb W (4), programmed between 100° and 200°.

#### Instrumentation

Measurements of enzyme activities were done using either a Gilford recording spectrophotometer or a Beckman DU spectrophotometer. Absorption spectra were determined using a Beckman DK2 spectrophotometer. NMR spectra in dimethyl sulfoxide- $d_6$  were obtained with a Varian Associates HA-100 spectrophotometer using tetramethylsilane as an internal standard. Gas-liquid chromatography was performed with a model 5750 Hewlett Packard gas chromatograph equipped with a flame-ionization detector.

## Results

### Growth of the Organism

The organism grew well with L-tyrosine as the sole carbon and nitrogen source. Because of the low solubility of tyrosine, gluconate was added to the growth medium to increase the yield of cells. When gluconate was present, together with additional nitrogen source, tyrosine was not utilized until after the gluconate had become exhausted. Cells quickly lost activity toward tyrosine and *p*-hydroxyphenylpyruvate when the growth medium became depleted of tyrosine. Best results were obtained using a medium containing 0.1% tyrosine and 0.1% gluconate, a standard inoculum, and an 18-h growth period. The organism grew readily with 0.2% *p*-hydroxyphenylacetate, L-phenylalanine, phenylacetate, but poorly with *o*-, or *m*-hydroxyphenylacetate. During growth on tyrosine, or *p*-hydroxyphenylacetate, the medium became yellow. Spectral examination of the yellow medium at acid and alkaline values of pH indicated the production of CHMS in the medium. Ether extracts of the acidified medium after growth on L-tyrosine contained phenolic compounds identical with *p*-hydroxyphenylacetate acid and 3,4-dihydroxyphenylacetic acid in thin-layer and gas-liquid chromatography studies.

### Studies with Intact Cells

Cells grown on tyrosine rapidly consume oxygen in the presence of L-tyrosine, *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, *m*-hydroxyphenylacetate, and 3,4-dihydroxyphenylacetate (Table 1). Homogentisate was not utilized by cells grown on any of the carbon sources shown in Table 1. The rate of oxygen consumption due to the addition of *p*-hydroxyphenylacetaldehyde varied inconsistently with different cell preparations. These results, together with later results obtained using cell extracts, make *p*-hydroxyphenylacetaldehyde an unlikely intermediate in the degradation of tyrosine. Cells grown with gluconate as the sole carbon source are not adapted to the utilization of any of the compounds listed in Table 1. The results suggest that the catabolism of tyrosine occurs by a pathway involving 3,4-dihydroxyphenylacetate as a key intermediate as shown in Fig. 1. A similar pattern of substrate utilization was obtained with cells adapted to the utilization of *p*-hydroxyphenylpyruvate. Cells grown on *p*-hydroxyphenylacetate or 3,4-dihydroxyphenyl-

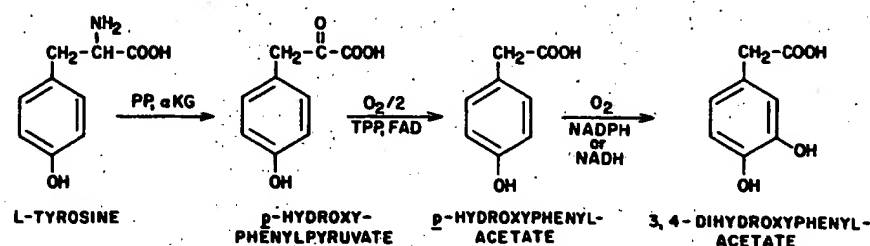
TABLE 1. Consumption of oxygen by *Arthrobacter* (PRLW 15)

Substrate	Relative rate <sup>a</sup> by cells grown on various carbon sources			
	Tyrosine	<i>p</i> -Hydroxyphenylpyruvate	<i>p</i> -Hydroxyphenylacetate	3,4-Dihydroxyphenylacetate
L-Tyrosine	100	72	1	0
<i>p</i> -Hydroxyphenylpyruvate	116	100	28	45
<i>p</i> -Hydroxyphenylacetate	107	104	100	117
<i>m</i> -Hydroxyphenylacetate	105	103	100	104
3,4-Dihydroxyphenylacetate	90	98	93	100
<i>p</i> -Hydroxyphenylacetaldehyde	92 <sup>b</sup>	16	103 <sup>b</sup>	—
D,L-Fluorotyrosine	48	—	—	—
D,L- <i>p</i> -Hydroxyphenylacetate	38	—	—	—
Phenylpyruvate	44	—	—	—
L-Phenylalanine	8	—	—	—
3,4-Dihydroxyphenylalanine	20	—	—	—

NOTE: The following compounds had relative rates less than 10: D,L-phenylalanine, D-tyrosine, phenylacetate, *o*-hydroxyphenylacetate, homogentisate, tyramine, dopamine, *p*-fluorophenylalanine, *o*-, *m*-, *p*-hydroxybenzoate, benzoate, protocatechuic acid, catechol.

<sup>a</sup>Relative rate is the rate of oxygen consumption compared to the rate obtained in the presence of the growth substrate (=100). All values are corrected for endogenous consumption of oxygen. Compounds not tested are indicated by —.

<sup>b</sup>The activity of cells towards this compound varied with different cell preparation.

FIG. 1. The oxidation of L-tyrosine to 3,4-dihydroxyphenylacetate by *Arthrobacter* sp (PRL W19).

acetate are adapted for the utilization of both compounds, but not tyrosine or *p*-hydroxyphenylpyruvate.

#### Detection of Enzymes in Extracts

##### (a) Tyrosine Aminotransferase (EC 2.6.1.5)

Tyrosine aminotransferase was readily detected by the methods of Lin *et al.* (19) or Diamondstone (10) in extracts prepared with buffer A (Table 2). The reaction required the specific addition of  $\alpha$ -ketoglutarate; oxalate, pyruvate, or glyoxylate could not replace  $\alpha$ -ketoglutarate as the amine acceptor. The addition of pyridoxal phosphate increased the rate of reaction. The addition of diethyldithiocarbamate ( $4 \times 10^{-3}$  M), an inhibitor of *p*-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.5), had no effect on the reaction. L-Phenylalanine effectively replaced tyrosine in the reaction system when tested by the method of Lin *et al.* (19).

##### (b) *p*-Hydroxyphenylpyruvate Oxidase

Under the conditions used for measuring

tyrosine aminotransferase, the product of the reaction did not disappear at a significant rate. However, extracts prepared in buffers B or C, and supplemented with thiamine pyrophosphate,  $\text{Mg}^{2+}$ , FAD, and reduced glutathione catalyzed a rapid disappearance of *p*-hydroxyphenylpyruvate. The enzyme activity could be precipitated from crude extracts, after treatment with 0.1 volumes of 2% protamine sulfate by the addition of ammonium sulfate without appreciable loss in activity and resulted in a two-fold increase in specific activity. Most of the activity precipitated between 40 and 55% ammonium sulfate saturation. Attempts to increase enzyme purity by chromatography on Sephadex or treatment with calcium phosphate gel resulted in large and variable losses in activity without appreciable changes in specific activity. Crude extracts and ammonium sulfate preparations could be stored at  $-20^\circ\text{C}$  for 4 weeks without appreciable loss in activities. However, at about  $0^\circ\text{C}$  (ice bath) such preparations lost about 10% of the activity per hour. The rate of activity loss

TABLE 2. Tyrosine aminotransferase

Reaction mixture changes		
Deletions	Additions	% of control <sup>a</sup>
Nil	12 $\mu$ mol diethyldithiocarbamate ( $4 \times 10^{-3}$ M)	95
Pyridoxalphosphate	Nil	66
$\alpha$ -Ketoglutarate	Nil	0
$\alpha$ -Ketoglutarate	30 $\mu$ mol oxalacetate	0 <sup>b</sup>
$\alpha$ -Ketoglutarate	30 $\mu$ mol glyoxylate	0
$\alpha$ -Ketoglutarate	30 $\mu$ mol $\alpha$ -ketobutyrate	0
$\alpha$ -Ketoglutarate	30 $\mu$ mol pyruvate	0.3
L-Tyrosine	19.2 $\mu$ mol L-phenylalanine	108 <sup>b</sup>

<sup>a</sup>The control reaction contained 19.2  $\mu$ mol L-tyrosine, 0.12  $\mu$ mol pyridoxalphosphate, 30  $\mu$ mol  $\alpha$ -ketoglutarate, 0.1 ml crude extract (0.6 mg protein) in 3.0 ml 0.2 M potassium phosphate, pH 7.3. The reaction was started by the addition of  $\alpha$ -ketoglutarate, incubated for 20 min, and stopped by the addition of 0.2 ml 10 N NaOH. The amount of *p*-hydroxyphenylpyruvate formed was measured at 331 nm according to Diamondstone (10).

<sup>b</sup>This was assayed by the method of Lin *et al.* (19).

TABLE 3. Requirements for maximum *p*-hydroxyphenylpyruvate oxidase activity

Changes in reaction mixture		
Deletions	Additions	% of control <sup>a</sup>
4 $\mu$ mol TPP	Nil	40
2 $\mu$ mol TPP	"	94
10 $\mu$ mol MgCl <sub>2</sub>	"	71
5 $\mu$ mol MgCl <sub>2</sub>	"	88
0.01 $\mu$ mol FAD	"	32
2 $\mu$ mol GSH	"	31
1 $\mu$ mol GSH	"	85
200 $\mu$ mol phosphate	200 $\mu$ mol arsenate	91
4 $\mu$ mol TPP,		
200 $\mu$ mol phosphate	200 $\mu$ mol arsenate	0
10 $\mu$ mol MgCl <sub>2</sub>	10 $\mu$ mol MnCl <sub>2</sub>	100 <sup>b</sup>

<sup>a</sup>The control reaction mixture contained in a final volume of 3.0 ml, 200  $\mu$ mol phosphate, pH 7.5, 4  $\mu$ mol TPP, 10  $\mu$ mol MgCl<sub>2</sub>, 0.01  $\mu$ mol FAD, 2  $\mu$ mol GSH, 0.2  $\mu$ mol *p*-hydroxyphenylpyruvate, and about 0.1 mg protein from a fraction obtained by ammonium sulfate precipitation and treated with calcium phosphate.

<sup>b</sup>Tris was used in place of phosphate to minimize precipitation of Mn<sup>2+</sup> salts.

was greater after dilution with buffer, which was required for enzyme assays, and amounted to at least three times the rate of the undiluted enzyme. When diluted with buffer containing ammonium sulfate (0.5–1.0 M) the enzyme activity was stable for at least 4 hours. Other salts such as NaCl, Na<sub>2</sub>SO<sub>4</sub>, or MgSO<sub>4</sub> were also effective in stabilizing the activity. Maximum stability of the activity was found between pH 7.0 and 7.6.

Because of the difficulty in stabilizing enzyme activity, attempts to purify the enzyme were not satisfactory. The properties of the enzyme were therefore examined using ammonium sulfate preparations. Maximum activity was found in a narrow range around pH 7.5 in phosphate buf-

fer, and required the addition of TPP, Mg<sup>2+</sup>, FAD, and GSH (Table 3). No enzyme preparations were obtained which required the specific addition of any of the cofactors for activity. The rates of reactions were faster in phosphate buffer than Tris, bicine, 2(*N*-morpholino)ethane sulfonic acid (MES), *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid HEPES, glycine, borate, or arsenate. The reaction was strongly inhibited in the absence of added TPP when arsenate replaced phosphate as the buffer. The results suggested that phosphate might play a significant role in the reaction mechanism, which was prevented in the presence of arsenate. However, attempts to obtain an enzyme preparation by fractionation in Tris buffer which had a



specific phosphate requirement and to detect the formation of *p*-hydroxyphenylacetylphosphate by trapping experiments with hydroxylamine (20) were unsuccessful. The addition of  $Mn^{2+}$  effectively replaced  $Mg^{2+}$ , but other cations such as  $Cu^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$ ,  $Zn^{2+}$  were without effect or were inhibitory. EDTA was a potent inhibitor of the reaction, but other metal chelators, including diethyldithiocarbamate, were ineffective. The failure of diethyldithiocarbamate to inhibit the reaction is significant, since the oxidation of *p*-hydroxyphenylpyruvate to homogentisate by *p*-hydroxyphenylpyruvate hydroxylase is inhibited by this compound. FAD could not be replaced by  $NAD^+$ ,  $NADP^+$ , or flavine mononucleotide (FMN). The addition of reduced GSH could be replaced by Cleland's reagent (dithiothreitol), cysteine, or mercaptoethanol.

Among several  $\alpha$ -keto acids tested including pyruvate, only phenylpyruvate and glyoxylate were found to be substrates for the reaction. The relative rates of reaction for *p*-hydroxyphenylpyruvate, phenylpyruvate, and glyoxylate were 1:0.84:0.19. The ratio of activities toward *p*-hydroxyphenylpyruvate and phenylpyruvate remained constant during various treatments of enzyme activity suggesting a single enzyme for both substrates. The same enzyme appears to be present in extracts grown on either L-tyrosine or L-phenylalanine. The enzyme was not effective in catalyzing the disappearance of *p*-hydroxyphenylacetaldehyde or phenylacetaldehyde, nor could evidence for an aldehyde dehydrogenase be obtained (12). It is concluded that the reaction does not involve an aldehyde as an intermediate.

(c) *p*-Hydroxyphenylacetate Hydroxylase

The presence of *p*-hydroxyphenylacetate hydroxylase was demonstrated in crude extracts by measuring the rate of oxidation of NADPH at 340 nm due to the addition of *p*-hydroxyphenylacetate. When *m*-hydroxyphenylacetate replaced *p*-hydroxyphenylacetate in the reaction mixture the rate was reduced by about 40%. Both NADH and NADPH were oxidized in the presence of *m*-hydroxyphenylacetate or *p*-hydroxyphenylacetate by ultracentrifuged extracts. When the coenzyme was NADPH, the reaction mixture became yellow, but the yellow color disappeared after addition of  $NAD^+$ . The enzyme appears to have properties similar to *p*-hydroxyphenylacetate-3-hydroxylase (EC

1.14.13.3) described by Adachi *et al.* (1), but is less coenzyme-specific.

(d) 3,4-Dihydroxyphenylacetate 2,3-Dioxygenase

The appearance of a compound having properties consistent with being CHMS in the growth medium and in experiments with cell suspensions or cell extracts indicated the presence of 3,4-dihydroxyphenylacetate-2,3-dioxygenase. The activity of the enzyme in extracts prepared in Tris buffer was easily determined by measuring the rate of production of CHMS from 3,4-dihydroxyphenylacetate at 380 nm. The enzyme could be heated at 55°C for 5 min or dialyzed against buffer for several hours at 0°C without appreciable loss in activity. Lyophilized preparations of the enzyme have been stored at 10°C without loss in activity after 8 months.

(e) CMHS Dehydrogenase

Crude extracts catalyzed the disappearance of CHMS in the presence of  $NAD^+$ .  $NADP^+$  could not replace  $NAD^+$ . The reaction rate was increased by the addition of  $Mg^{2+}$ . The enzyme activity was completely destroyed by heating at 55°C for 5 min.

(f) Succinic Semialdehyde Dehydrogenase

Ultracentrifuged extracts reduced  $NAD^+$  or  $NADP^+$  in the presence of succinic semialdehyde. The rate with  $NADP^+$  was about twice that with  $NAD^+$ . The product from a large-scale reaction mixture was isolated by ether extraction and identified as succinic acid by gas chromatography studies.

Manometric Studies with Extracts

Crude extracts prepared with buffer A, consumed 1 mol of oxygen per mole of 3,4-dihydroxyphenylacetate. When Tris buffer was replaced by 0.0125 *N*  $NaHCO_3$ , about 1 mol of carbon dioxide was released, indicating the formation of one equivalent of acid per mole of 3,4-dihydroxyphenylacetate. The oxidation of 3,4-dihydroxyphenylacetate resulted in the production of a compound which was yellow in alkaline solution and colorless in acidic solution. Isolation and characterization of the compound from a large-scale reaction mixture confirmed that the compound was  $\delta$ -carboxymethyl- $\alpha$ -hydroxymuconic semialdehyde (CMHS) (see later in Results). This result demonstrated the *meta* cleavage of 3,4-dihydroxyphenylacetate and the presence of 3,4-dihydroxyphenylacetate-2,3-



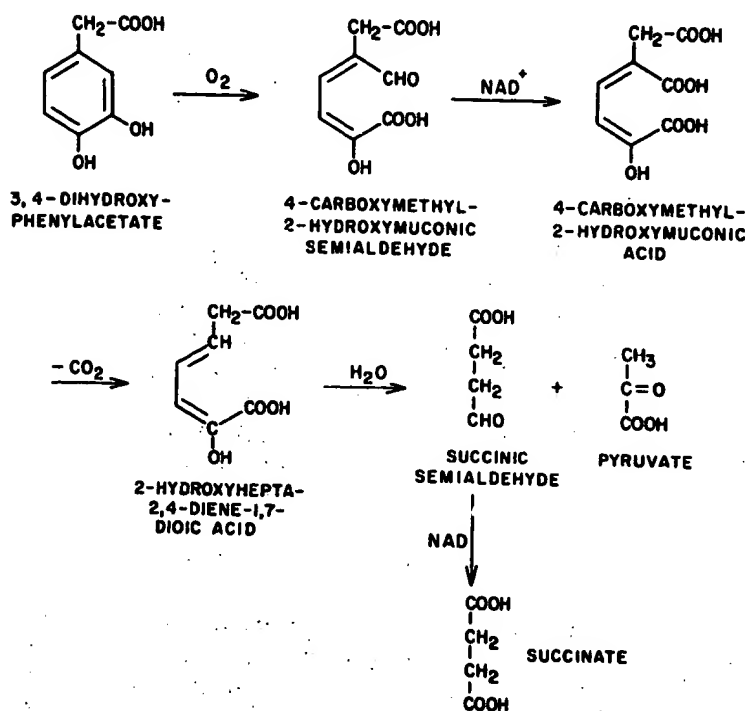


FIG. 2. The degradation of 3,4-dihydroxyphenylacetate by *Arthrobacter* sp. (PRL W19).

dioxygenase (EC 1.13.11.15) in the extracts. Ultracentrifuged extracts, dialyzed extracts, extracts heated at 55°C for 5 min, or preparations obtained by ammonium sulfate fractionation gave similar results.

CHMS did not accumulate from 3,4-dihydroxyphenylacetate when  $NAD^+$  was added to the extracts. Under these conditions the consumption of oxygen increased to nearly 2 mol of oxygen per mole of 3,4-dihydroxyphenylacetate, and about 1 mol of carbon dioxide per mole of substrate was produced. When 0.0125 *M*  $NaHCO_3$  replaced Tris buffer, the production of carbon dioxide increased to nearly 2 mol per mole of substrate.  $NADP^+$  could not replace  $NAD^+$  in the reaction. Since crude extracts contain high levels of NADH oxidase activity, it appears that oxygen is being consumed by the oxidation of NADH. The foregoing observations suggest that 3,4-dihydroxyphenylacetate is oxidized by 1 mol of oxygen to form CHMS, which is then oxidized by a  $NAD^+$ -specific dehydrogenase with the concomitant release of carbon dioxide (See Fig. 2). The dehydrogenase activity is destroyed in extracts heated at 55°C for 5 min.

Attempts to study the oxidation of CHMS by extracts in the Warburg apparatus were not

satisfactory. Under conditions which rapidly oxidized 3,4-dihydroxyphenylacetic acid in the presence of  $NAD^+$ , the consumption of oxygen and production of carbon dioxide proceeded slowly, and did not go to completion when compared to results obtained with 3,4-dihydroxyphenylacetate, and as judged by the incomplete disappearance of yellow color. It appeared that under these conditions CHMS interfered with enzyme activity.

Crude extracts supplemented with NADPH consumed about 2 mol of oxygen per mole of *p*-hydroxyphenylacetate or *m*-hydroxyphenylacetate, and the reaction mixture became yellow because of the formation of CHMS. Under these conditions NADH was a poor substitute for NADPH. However, with ultracentrifuged extracts, both NADPH and NADH were effective. The reaction mixtures became yellow only when NADPH was used. These results show that *p*-hydroxyphenylacetate and *m*-hydroxyphenylacetate are oxidized through 3,4-dihydroxyphenylacetate to CHMS (Fig. 1).

Under the conditions described above, extracts failed to oxidize tyrosine or *p*-hydroxyphenylpyruvate. However, when extracts were prepared using buffers B and C (containing

thiamine pyrophosphate,  $\text{MgCl}_2$ , and mercaptoethanol) and supplemented with thiamine pyrophosphate,  $\text{MgCl}_2$ , FAD, and reduced glutathione, *p*-hydroxyphenylpyruvate was oxidized with the consumption of 0.5 mol of oxygen and the production of 1 mol of carbon dioxide per mole of substrate (Fig. 3). Under these conditions, *p*-hydroxyphenylacetate was not appreciably oxidized, but when NADPH was added to the reaction mixtures containing *p*-hydroxyphenylpyruvate, the consumption of oxygen exceeded 2 mol of oxygen per mole of substrate. This is the expected result if the product of the reaction with *p*-hydroxyphenylpyruvate is *p*-hydroxyphenylacetate.

The addition of ethanol (400  $\mu\text{mol}$ ) and catalase (1 mg) to reaction mixtures containing *p*-hydroxyphenylpyruvate resulted in a twofold increase in oxygen consumption, and suggested the formation of hydrogen peroxide during the reaction. Additional evidence for the formation of hydrogen peroxide during the reaction was obtained by using *o*-dianisidine reagent (32).

The further supplementation of *p*-hydroxy-

phenylpyruvate-active reactions with  $\alpha$ -ketoglutarate and pyridoxal phosphate resulted in the consumption of oxygen due to the addition of tyrosine.

*p*-Hydroxyphenylacetaldehyde was not oxidized under conditions in which tyrosine, *p*-hydroxyphenylpyruvate, and *p*-hydroxyphenylacetate were active. From the failure to find *p*-hydroxyphenylacetaldehyde to be an active substrate in spectrophotometric and manometric studies with cell extracts, it is concluded that it is not an intermediate in the catabolism of tyrosine.

#### Identification of Products of Reactions with Cell Extracts

##### (a) The Product from Tyrosine

The reaction of tyrosine with cell extract, as described in Methods and Materials, resulted in the formation of 50  $\mu\text{mol}$  of *p*-hydroxyphenylpyruvate according to assay methods of Diamondstone (10) and Lin *et al.* (19). The ultraviolet spectrum of the enol-borate complex of the product had an absorption maximum at 308 nm (19) and was identical with the spectrum of authentic *p*-hydroxyphenylpyruvate. Examination of the product and its 2,4-dinitrophenylhydrazone by paper and thin-layer chromatography provided further evidence for the identity of the product of the reaction with tyrosine as *p*-hydroxyphenylpyruvate.

##### (b) The Product from *p*-Hydroxyphenylpyruvate

The ether extract from a reaction mixture of *p*-hydroxyphenylpyruvate contained a single compound with properties identical with *p*-hydroxyphenylacetate when examined by paper and thin-layer chromatography in a number of solvent systems (Table 4), and the same retention time by gas-liquid chromatography as *p*-hydroxyphenylacetate when run as the silyl derivative (4). Reaction mixtures containing no *p*-hydroxyphenylpyruvate contained no detectable compounds in the ether extracts.

##### (c) The Products from 3,4-Dihydroxyphenylacetate

The oxidation of 3,4-dihydroxyphenylacetate as described in Materials and Methods for the preparation of CHMS resulted in a linear disappearance of 3,4-dihydroxyphenylacetate and a stoichiometric production of CHMS. During the reaction, about 1.5 mol of NaOH per mole of 3,4-dihydroxyphenylacetate were required.

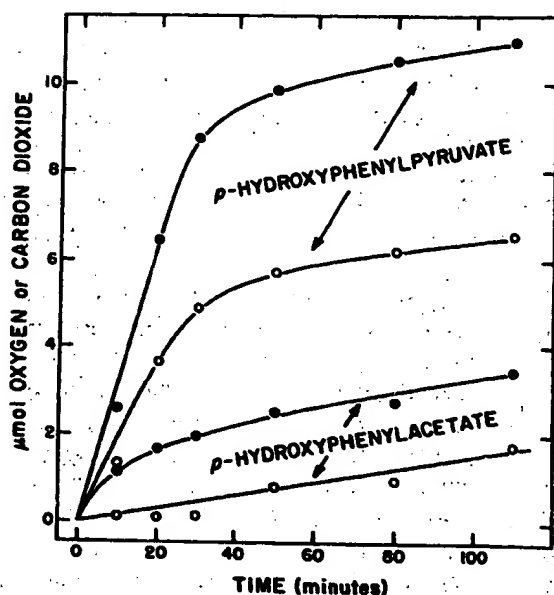


FIG. 3. Consumption of oxygen (○—○) and production of carbon dioxide (●—●) from *p*-hydroxyphenylpyruvate and *p*-hydroxyphenylacetate by crude extracts of *Arthrobacter*. The reaction mixtures contained 200  $\mu\text{mol}$  of phosphate buffer, pH 6.5, 4  $\mu\text{mol}$  of thiamine pyrophosphate, 10  $\mu\text{mol}$  of  $\text{MgCl}_2$ , 0.01  $\mu\text{mol}$  of flavine adenine dinucleotide, 2  $\mu\text{mol}$  of reduced glutathione, 10  $\mu\text{mol}$  of substrate, 1.0 ml of crude extract (10 mg protein) in a final volume of 3.0 ml. Results are corrected for endogenous oxygen consumption.

TABLE 4. Chromatography of reaction product of *p*-hydroxyphenylpyruvate

Solvent system <sup>a</sup>	Product	<i>R<sub>f</sub></i>	
		<i>p</i> -Hydroxyphenyl-acetic acid	<i>p</i> -Hydroxyphenyl-pyruvate
J W	0.58	0.58	Streaks
J TL	0.65	0.65	Streaks
H TL	0.36	0.36	0.24, 0.08
I TL	0.33	0.33	0.27

<sup>a</sup>See Materials and Methods for description.

The consumption of NaOH in excess of 1 mol per mole of 3,4-dihydroxyphenylacetate was assumed to be due to the enolizable property of the  $\alpha$ -keto group of CHMS. The yield of product from 336 mg 3,4-dihydroxyphenylacetate was about 200 mg. The product had the same spectral properties in acid and alkaline solution as described for CHMS (1, 6), and the pyridine dicarboxylic acid which resulted after treatment with ammonia, followed by oxidation with potassium permanganate (1, 6) had the same melting point, ultraviolet spectrum, and nuclear magnetic resonance spectrum as authentic pyridine-2,5-dicarboxylic acid. The characterization studies established the structure of the product as CHMS.

When reaction mixtures containing 3,4-dihydroxyphenylacetate and  $\text{NAD}^+$  were allowed to go to completion and then treated with 2,4-dinitrophenylhydrazine, a compound was isolated which agreed in properties with pyruvate-2,4-dinitrophenylhydrazone by paper and thin-layer chromatography. Ether extracts of the acidified reaction mixtures contained a compound having properties identical with succinic acid when examined by paper and thin-layer chromatography in several solvent systems. The presence of pyruvate could not be clearly recognized in these extracts. Gas-liquid chromatography of silyl derivatives of compounds isolated by ether extraction of the acidified reaction mixture showed the presence of succinate and pyruvate. A comparison of the recoveries of acids from a reaction mixture initially containing 10  $\mu\text{mol}$  of 3,4-dihydroxyphenylacetate with a reaction mixture containing 10  $\mu\text{moles}$  of succinate and 10  $\mu\text{mol}$  of pyruvate showed good agreement. This result indicates the production of 1 mol of succinate and 1 mol of pyruvate from 1 mol of 3,4-dihydroxyphenylacetate (Fig. 2).

#### Stoichiometry in the Oxidation of 3,4-Dihydroxyphenylacetate

The oxidation of 3,4-dihydroxyphenylacetate to succinate and pyruvate should require 2 mol of  $\text{NAD}^+$ . Support for this relationship was obtained by following the reaction at 340 nm (Fig. 4) using ultracentrifuged extracts. In the absence of added  $\text{NAD}^+$ , only CHMS is formed and the absorbance at 340 nm is due to the presence of this compound. When one equivalent of  $\text{NAD}^+$  is added, (curve 2) the reaction mixture becomes yellow, indicating insufficient  $\text{NAD}^+$  to oxidize completely the CHMS. The absorbance at 340 nm is due to

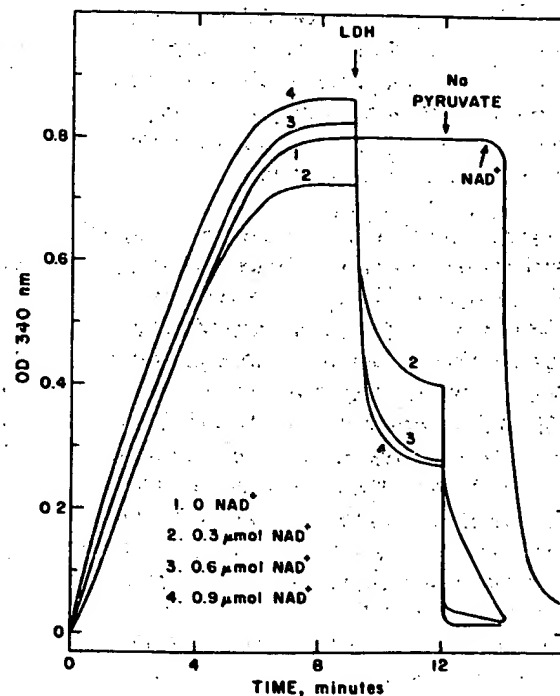


FIG. 4. The oxidation of 0.3  $\mu\text{mol}$  3,4-dihydroxyphenylacetate by ultracentrifuged extracts of *Arthrobacter*. At the times indicated by arrows, lactic acid dehydrogenase (LDH) or 10  $\mu\text{mol}$  pyruvate was added.

both NADH and residual CHMS. Two equivalents (curve 3) and 3 equivalents (curve 4) of  $\text{NAD}^+$  oxidize all the CHMS, as judged visually by the absence of yellow color. The absorbance at 340 nm is due to the presence of NADH. The addition of lactic acid dehydrogenase (LDH) to the reaction mixtures results in a decrease in absorbance as a result of the reaction of the pyruvate with NADH. The decrease in reaction mixtures with 2 and 3 equivalents of  $\text{NAD}^+$  (curves 3 + 4) are about 50%, while decrease with 1 equivalent of  $\text{NAD}^+$  (curve 2) is less than 50%. The regeneration of  $\text{NAD}^+$  causes complete disappearance of the yellow color in reaction 2. The addition of pyruvate to the reaction mixtures oxidizes NADH. These observations suggest that 2 mol of  $\text{NAD}^+$  are required for the complete oxidation of 3,4-dihydroxyphenylacetate by ultracentrifuged extracts. One mole of  $\text{NAD}^+$  was required for the oxidation of CHMS, the other mole for the oxidation of succinic semialdehyde.

The evidence for succinic semialdehyde being an intermediate in the catabolism of tyrosine is based on the presence of succinic semialdehyde dehydrogenase in extracts. Attempts to detect succinic semialdehyde in reaction mixtures gave inconclusive results.

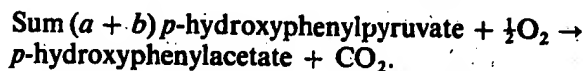
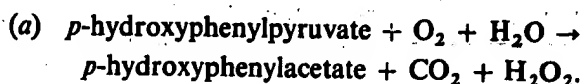
### Discussion

While this work was in progress Sparins *et al.* (26) described the bacterial degradation of 3,4-dihydroxyphenylacetic acid by strains of *Acinetobacter* and *Pseudomonas* and have identified the intermediates in the degradative pathway. The results obtained in the present study for the degradation of 3,4-dihydroxyphenylacetic acid by a strain of *Arthrobacter* are consistent with those found by Sparins *et al.* (26). The pathway for the degradation of 3,4-dihydroxyphenylacetic acid is shown in Fig. 2.

The results in the present paper show that a strain of *Arthrobacter* degrades tyrosine by a pathway involving 3,4-dihydroxyphenylacetate, rather than homogentisate as a key intermediate. Such a pathway was indicated in earlier studies by Kunita (18). More recently, while this paper was being prepared for publication, Sparins and Chapman (25) described the pathway for several gram-positive organisms. Thus the microbial catabolism of tyrosine by a pathway involving 3,4-dihydroxyphenylacetate may be more widespread than was originally thought.

The degradative pathway for tyrosine de-

scribed in this paper differs from that described by Sparins and Chapman (25) in that *p*-hydroxyphenylacetaldehyde is not postulated to be an intermediate. Evidence is presented that *p*-hydroxyphenylpyruvate is converted to *p*-hydroxyphenylacetate with the consumption of 0.5 mol of oxygen and the production of 1 mol of carbon dioxide per mole of substrate. Hydrogen peroxide is also produced during the reaction. Because of the presence of catalase in the extract preparations, the stoichiometry of the reaction may be illustrated by the following equations:



However, a non-enzymatic oxidation of *p*-hydroxyphenylpyruvate by hydrogen peroxide may also take place, resulting in the same stoichiometric relationships.

The formation of *p*-hydroxyphenylacetate from *p*-hydroxyphenylpyruvate has been reported by Fellman *et al.* (11) in studies with rat liver homogenates, but the conversion may be non-enzymatic. Other studies (3, 12, 24) have shown the conversion of phenylpyruvate to phenylacetate, but the process requires two enzymatic steps and involves phenylacetaldehyde as an intermediate. In the present study enzyme preparations do not catalyze the disappearance of *p*-hydroxyphenylacetaldehyde or phenylacetaldehyde, nor was evidence for *p*-hydroxyphenylacetaldehyde or phenylacetaldehyde dehydrogenase found in cell extracts. It is suggested that the conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxyphenylacetate may occur by a phosphate-linked oxidative decarboxylation analogous to the reaction of pyruvate oxidase (EC 1.2.3.3.) on pyruvate (14). Such a reaction does not appear to have been previously described.

The first two enzymes in the catabolic pathway, tyrosine aminotransferase and *p*-hydroxyphenylpyruvate oxidase were equally present in extracts of cells grown on either L-tyrosine or L-phenylalanine. However, the product of the reactions with tyrosine, *p*-hydroxyphenylacetate, is not appreciably oxidized by cells, or extracts of cells grown on phenylalanine, and the product

of the reactions with phenylalanine, phenylacetate, is not oxidized by cells or extracts of cells grown on tyrosine. Thus, for this organism the first two steps in the metabolism of L-tyrosine and L-phenylalanine are similar, but the metabolic fate of phenylacetate formed from L-phenylalanine is uncertain.

### Acknowledgements

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